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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

During transformation, ribosome biogenesis increases concurrently with the size and number of nucleoli. The U3 snoRNP is involved in the cleavage of pre-rRNA and 40S preribosomal particle assembly and contains a number of proteins, including Sof1 and fibrillarin, whose roles in the complex are unclear. Depletion of yeast Sof1 inhibits pre-rRNA processing, consistent with a functional role in the U3 snoRNP.

Our research focuses on characterizing the role of human Sof1 (hSof1) in ribosome biogenesis by examining its localization and dynamics in live cells and its interactions. hSof1, like fibrillarin, localizes to both the nucleolus and nucleoplasm. However, unlike fibrillarin, hSof1 is also in the granular component of nucleoli and responds differently to the inhibition of the transcription of pre-rRNA. In addition, hSof1-GFP also exhibits a higher nuclear mobility than fibrillarin-GFP and is a nucleocytoplasmic shuttling protein. hSof1p is associated with cytoplasmic ribosomal subunits, indicating that the protein may remain associated with preribosomal particles after they leave nucleoli and nuclei. We are currently mapping the domains of hSof1, attempting functional disruption, and plan to determine its tertiary structure. These experiments will clarify the roles of Sof1 in ribosome biogenesis and contribute to our understanding of ribosome biogenesis as a whole.

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INTRODUCTION

Breast cancer is a widespread disease with a high morbidity rate and the molecular and cellular changes that act in its development and progression are not yet well understood. As the functions of these cells change, so does the molecular and structural makeup of their nuclei to facilitate these changes. One of the most easily visible nuclear changes that take place during transformation is an increase in the size and shape of nucleoli, a long-utilized diagnostic marker (1, 2, 3) that changes concurrently with an increase in ribosome biogenesis (4). In most cells, ribosome biogenesis is closely controlled and requires a large number of trans-acting factors that act to transcribe, process, and assemble preribosomal particles in the nucleolus (5, 6). The U3 small nucleolar ribonucleoprotein (snoRNP) is such a trans-acting nucleolar complex that acts in the cleavage of pre-ribosomal RNA (pre-rRNA) and in preribosomal particle assembly. The U3 snoRNP consists of the U3 small nucleolar RNA (snoRNA) and a number of associated proteins, including fibrillarin and Sof1p (5). Although fibrillarin is believed to be a methyltransferase (5), the function of Sof1p in this complex is unclear. A knockout of ySof1p is lethal while depletion inhibits 35S pre-rRNA processing, consistent with a functional role in the U3 snoRNP (7). Only limited studies on Sof1p have been done in yeast and none done on the human isoform (hSof1p).

BODY
RESEARCH PROGRESS

The direction of the research focus has been altered significantly from that outlined in the original statement of work and now focuses on the characterization of hSof1p. Sof1p consists of a unique amino-terminus, seven central WD40 repeats, and a unique, charged carboxy-terminus (Fig 1A). Though there is a no identifiable nuclear localization signal, ySof1p was found predominantly in the nucleoli and nucleoplasm. Since only limited characterization of Sof1p had been done in yeast, the main goal of my thesis research is to characterize the structure and function of the human isoform. My research on hSof1p has been divided into five main goals: (1) Identification and cloning of unknown nucleolar antigen and production of antibodies (completed at the time of the 2001 report), (2) Localization and dynamics studies of both endogenous and transfected hSof1p, (3) Characterization of hSof1p interactions, (4) Functional characterization of hSof1p, and (5) Determination of hSof1p-tertiary structure.

2. Localization and dynamics studies of both endogenous and transfected hSof1p

This task was partially completed at the time of last year's report and has progressed since that time. Localization and dynamics studies of hSof1p are necessary to determine the mode of action of the protein. These were initially performed using transfection of tagged constructs and were duplicated, when possible, using anti-Sof1p antibodies to recognize endogenous protein. The full-length hSof1p clone was tagged with GFP or FLAG, transfected into HeLa cells, and immunofluorescence was used to analyze their localization. Both tagged proteins were partially colocalized with fibrillarin in nucleoli, exhibited a strong nucleoplasmic label, and a weak cytoplasmic label (Fig 1B, top panel). Similar results were obtained using antibodies against endogenous hSof1p (Fig 1B, middle panel).

Deletion analyses were preformed to determine the domains necessary for hSof1p localization. A panel of over 20 mutants were made by fusing deletions from the amino- and carboxy- termini and deletions from the middle of the protein to GFP or a FLAG tag. By transfecting these constructs into HeLa cells, it was found that the 93 amino acid carboxy-terminus is sufficient for wild-type localization. Deletions are now being constructed to narrow down the specific sequence necessary for localization.

Last year, it was also determined that the localization of hSof1p was dependent upon the transcription of nascent rRNA. When this transcription is inhibited, most rRNA transcriptional, processing, and assembly factors (including fibrillarin) separate from the granular component of the nucleolus, the site where the final assembly of preribosomal particles takes place (Fig 1B, bottom panel). In contrast, hSof1p remains localized predominantly to the granular component (Fig 1B, bottom panel). These results together suggest that the nucleolar localization of hSof1p is due to its binding directly or indirectly to pre-rRNA, and that it may be associated with the processing and assembly of preribosomal particles in steps further downstream than those participated in by the U3 snoRNP.

In addition to the analyses of the steady-state intracellular localization of hSof1p, we have also examined the dynamics of hSof1p in living cells, as discussed in detail in our last report. Briefly, fluorescence recovery after photobleaching (FRAP) assays using hSof1p-GFP showed that the nucleolus-associated protein exchanges rapidly with the pool in the nucleoplasm. In addition, heterokaryon assays showed that hSof1p-GFP shuttles between nuclei and the cytoplasm, indicating that its function may require movement from nucleoli to the cytoplasm.

(3) Characterization of hSof1p interactions

We have been interested in identifying cellular factors that interact with Sof1p with hopes of gaining insight into its function. Since Sof1p is part of a multiprotein snoRNP complex and other WD40-repeat containing proteins have been shown to form multiprotein complexes (8), it is likely that it makes one or more functional interactions. These interactions are currently being explored using cofractionation experiments, immunoprecipitations, and recombinant protein pull-down assays.

Because hSof1p colocalizes with preribosomal particles in the granular component of nucleoli and is a nucleocytoplasmic shuttling protein, we tested whether it remains associated with ribosomes in the cytoplasm. Endogenous and GFP-labeled hSof1p have been found in purified cytoplasmic ribosomes from HeLa cells cofractionates with cytoplasmic 40S ribosomal subunits and 40S protein RPS6 in sucrose gradients (Figure 2A). So far we have been unable to completely separate the 40S and 60S subunits, leaving the possibility that some hSof1p is associated with the 60S subunit as well. The cofractionation of hSof1p and cytoplasmic 40S subunits is abolished upon treatment of lysates with RNAse, as would be expected if hSof1p is associated with the 40S subunit. Experiments using lysates of HeLa transfected with the aforementioned hSof1p-GFP deletion mutants have shown that the C-terminus is required for this association and the domain(s) required is currently being elucidated.

More direct evidence of the association of hSof1p with cytoplasmic ribosomes was obtained using immunoprecipitation experiments. Anti-hSof1p antibodies were used in immunoprecipitations from HeLa cytoplasmic lysates and the precipitates were analyzed by western blots against 40S subunit (RPS6) and 60S subunit (RPL7) proteins. These experiments showed that RPS6, but not RPL7, was specifically immunoprecipitated along with hSof1p, further supporting an *in vivo* association of hSof1p and the 40S subunit. Combined with other

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data, this implies that hSof1p may travel out to the cytoplasm with assembled ribosomal particles and could play roles in cytoplasmic assembly or function of ribosomes.

Further associations are currently being identified using immunoprecipitation and recombinant protein pull-down experiments. Immunoprecipitations are currently being scaled up to have sufficient, specific precipitates for protein sequencing. In the case of the pull-downs, we have been unable to produce tagged or untagged full-length hSof1p that is sufficiently soluble for the pull-down experiments and this appears to be due to its WD40 repeats. Current experiments are using affinity columns containing hSof1p amino- or carboxy- termini-GST fusion proteins. HeLa lysates are being run through these columns and specifically-bound proteins will be sequenced and used to identify interacting partners. Once interacting partners have been identified, further experiments will be designed to determine if the interactions are direct or indirect and what functional significance these interactions may have.

(4) Functional characterization of hSof1

The fourth focus of this project has been to identify the function of hSof1p by disrupting its activity. Since we cannot as directly manipulate the hSof1p gene as others did with ySof1p, we have attempted to affect its function *in vitro* using a cell-free translation system and *in vivo* using dominant-negative mutants, antibody injection, and small interfering RNAs (siRNAs).

Since hSof1p is associated with cytoplasmic ribosomes, we are currently exploring if hSof1p affects translation of these ribosomes *in vitro*. We are adding either anti-hSof1p antibodies or recombinant hSof1p protein to rabbit reticulocyte lysates and assaying any effects on translation by looking at S^{35}-Methionine labeled protein products. These experiments have just begun and we expect results in a month or two.

For the *in vivo* experiments, it was initially our hope that one of the GFP-tagged deletion constructs would act as a dominant-negative. Although a number of these constructs contain the majority of the protein and have completely different subcellular localization, none of these has resulted in the changes in nuclear morphology or redistribution of endogenous hSof1p or other nucleolar proteins that would be indicative of the interruption of ribosomal biogenesis.

We have attempted the cytoplasmic and nuclear injection of several different anti-hSof1p antibodies with the goal of disrupting hSof1p function. We have managed to obtain some disruption of nucleolar structure under some conditions, though these results are still preliminary. These experiments are ongoing and are still being optimized.

We have also attempted to disrupt hSof1p function *in vivo* using siRNA constructs, which should result in degradation of hSof1p mRNA and result in eventual depletion of the protein. Our original experiments utilized a synthetic RNA/DNA construct directed against a region in the amino-terminus of hSof1p mRNA. However, when this construct was transfected into HeLa cells, no reduction in hSof1p protein level or alteration of nucleolar structure was seen in the 72 hours post-transfection. The failure of these experiments was most likely due to the target site selection. We are currently producing siRNA constructs against two different regions of hSof1p mRNA using a plasmid-based system (Ambion). We hope to have these constructs ready within another month and will transfect these into HeLa cells. Hopefully targeting different regions of hSof1p mRNA will allow efficient inhibition of hSof1p protein translation and we can then monitor the effects by looking at nucleolar protein distribution and cytoplasmic ribosome profiles.

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(5) Determination of hSof1p-tertiary structure

The fifth approach to characterizing hSof1p is characterization of its tertiary structure. We have established a collaboration with the lab of Dr. Wayne Anderson to determine the tertiary structure of recombinant hSof1p using X-ray crystallography. However, as described above, full-length hSof1p with or without His or GST tags has proven to be insoluble in nondenaturing conditions. Attempts to do large scale preparations, denaturation, and refolding have not yielded sufficient protein for crystallization. We are still attempting to solubilize the protein in sufficient amounts for these experiments. If successful in doing this, then the elucidation of hSof1p structure should assist us in understanding how the protein functions.

Overall, these five primary goals have already increased our understanding of the role of hSof1p in ribosome biogenesis and further investigations should yield a more refined picture of its structure and function. These finding will contribute to our understanding of ribosome biogenesis and may provide new insight into how changes in ribosomal biogenesis are related to the transformed phenotype.

TRAINING PROGRESS

Significant advances in training have been made. As described above, the characterization of hSof1p has required the use of multiple techniques. These have included molecular biological (RT-PCR, expression cloning, deletion mutagenesis, siRNA), biochemical (protein expression and purification, immunoprecipitation, sucrose gradient fractionation), and cell biological (localization studies, nucleocytoplasmic shuttling assays, FRAP) approaches. Studies planned for the future will necessitate the continued use of these and other techniques.

Although I have finished all of my graduate coursework, I am continuing to stay up to date on the current research in the ribosomal, nuclear, and cancer fields. Besides reading current literature, I participate in a cancer biology journal club and attend both departmental and other lecture series. These activities keep me well informed on a broad range of biological research. Further education has taken place at several scientific meetings. I attended the December 2001 Annual Meeting of the American Society for Cell Biology (ASCB) and presented a poster on my thesis work. In addition, this year I participated in and presented a poster at the Era of Hope meeting and will present a poster at this December's annual ASCB meeting.

Apart from my thesis work, I have also contributed to three publications in collaboration with my labmates. I am second author on a research report that was published last December in Molecular Biology of the Cell which explored the functional dynamics of a perinucleolar compartment-associated splicing protein. I was also first author on a solicited minireview on the regulation of ribosome biogenesis that was published in and occupied the cover of FEBS letters last December. In addition, I am second author on a book chapter which reviews the present and future of nuclear makers of cancer. Within the six months, we expect to publish one or more papers on my thesis research. I am still pursuing a career in which I can explore the cell biology of cancer and am currently applying to medical schools so that I can accomplish my goals as an M.D./Ph.D.

KEY RESEARCH ACCOMPLISHMENTS

- Characterizing the localization and dynamics of hSof1p
- Characterizing the ribosomal association of hSof1p
- Ongoing characterization of hSof1p function *in vitro* and *in vivo*
- Producing full-length recombinant hSof1p

REPORTABLE OUTCOMES

1. Research Article: R. Kamath, D. J. Leary, and S. Huang. 2001. Nucleocytoplasmic Shuttling of Polypyrimidine Tract-binding Protein Is Uncoupled from RNA Export. Mol Biol Cell. 12:3808-3820.
2. Minireview: D. J. Leary and S. Huang. 2001. Regulation of Ribosome Biogenesis within the Nucleolus. FEBS Lett. 509(2):145-150.
3. Poster presentation at the Annual Meeting at the American Society of Cell Biology, December, 2001. Poster entitled "Characterization of hSof1p, Human Homolog of a Yeast U3 snoRNP Component"
4. Book Chapter: R. Kamath, D. J. Leary, and S. Huang. Nuclear Components and Tumor Markers. *In* Visions of the Nucleus – Eukaryotic DNA. P. Hemmerich and S. Diekmann, editors. American Scientific Publishers. In Review.
5. Poster presentation at the Era of Hope Meeting, September, 2002. Poster entitled "hSof1p, a U3 snoRNP component, is associated with cytoplasmic ribosomes."
6. Poster presentation at the Annual Meeting at the American Society of Cell Biology, December, 2002. Poster entitled "hSof1p, Human Homolog of a Yeast U3 snoRNP Component, is Associated with Cytoplasmic Ribosomes"

CONCLUSIONS

Significant progress has been made in characterizing the structure and function of hSof1p. This research has provided me with extensive laboratory experience and should yield several publications. In addition, my active involvement in journal clubs, scientific meetings, and writing have kept me well-informed of advances in my field. I believe that together these aspects of my doctoral training have and will continue to prepare me well for future research into the cell biology of cancer.

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A.

N-terminus   WD-40 Repeats   Chrarged C-Terminus
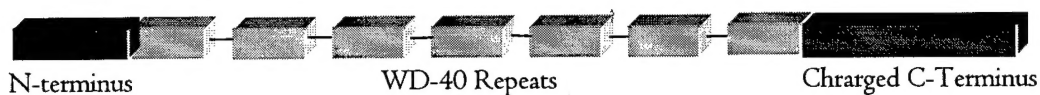
B.
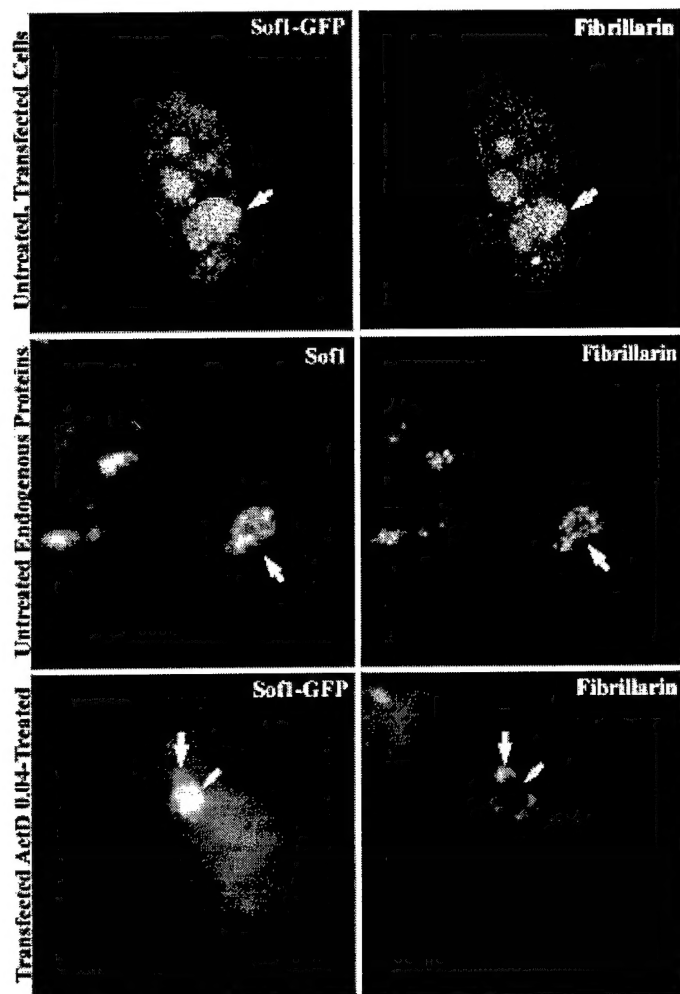
Figure 1. hSof1p is a WD40 protein which localizes to the fibrillar and granular regions of nuclei and whose localization is dependent upon transcription of nascent rRNA.

A. Schematic representation of the domain structure of hSof1p. The amino (N)-terminus and charged carboxy (C)-terminus flank seven WD40 repeats.

B. The localization of hSof1p-GFP is similar to that of the U3 snoRNP component fibrillarin. Both are concnetrated in nucleoli (top panel, arrows) and exhibit strong nucleoplasmic and some cytoplasmic stain. Endogenous protein labeling (middle panels) is similar. However, unlike fibrillarin, when RNA polymerase I is inhibited (bottom panels), hSof1p-GFP remains associated with the granular component of nucleoli (thin arrow) whereas fibrillain localizes primarily to the nucleolar caps (thick arrows). These results indicate that these proteins may be involved in different steps of ribosome biogenesis.
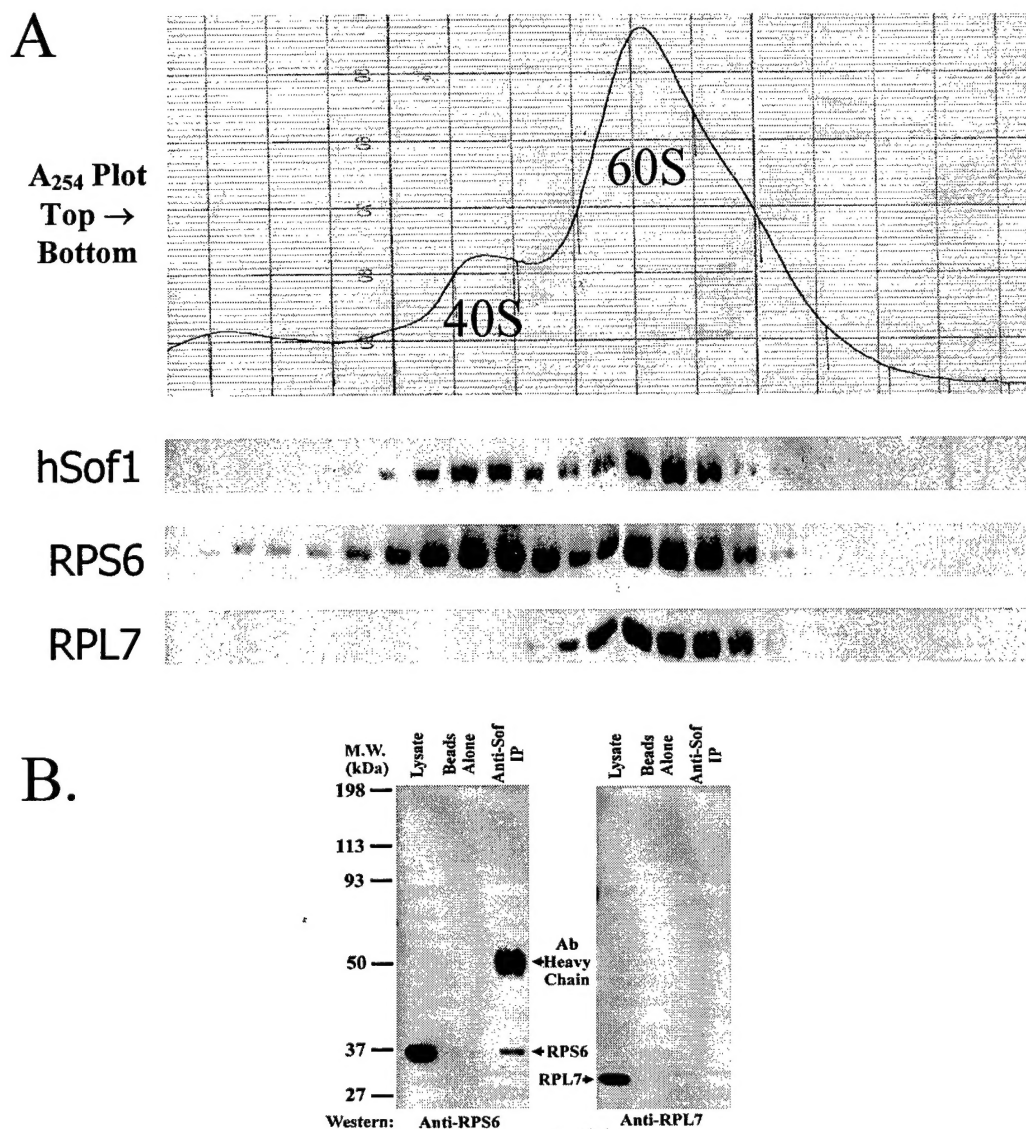
Figure 2 hSof1p is associated with cytoplasmic small (40S) ribosomal subunits

A. Cytoplasm was extracted from HeLa cells that were pretreated with puromycin to inhibit translation. This extract was separated through a 5-30% sucrose gradient that also contained puromycin. The gradint was fractionated while continuous A_{254} readings were taken. The fractions were concentrated, run on 12.5% SDS-PAGE gels, and westerns were done using antibodies against hSof1p (254), the small subunit protein S6 (RPS6), and the large subunit protein L7 (RPL7). The results indicate that RPS6 and the 40S peak in the gradient. hSof1p also partially cofractionated with RPL7, though RPS6 was also present in the 60S peak.

B. Cytoplasm was extracted from HeLa cells and immunoprecipitations were done using either anti-hSof1p antibody 255 linked to Protein A-sepharose beads or the Protein-A sepharose beads alone. The original lysate and the immunoprecipitations were run on 8% SDS-PAGE gels and westerns were done against the small subunit protein S6 (RPS6), and the large subunit protein L7 (RPL7). The results show that anti-Sof1p antibody specifically pulled down RPS6 (left panel) but not RPL7 (right panel). These results are consistant with an association between hSof1p and the small ribosomal subunit in the cytoplasm.